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Circulating Plasma Lipoproteins and Hepatic Ultrastructure in the Hibernating Thirteen-Lined Ground Squirrel, Spermophilus Tridecemlineatus

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CIRCULATING PLASMA LIPOPROTEINS AND HEPATIC ULTRASTRUCTURE IN THE HIBERNATING THIRTEEN-LINED GROUND SQUIRREL, *Spermophilus tridecemlineatus*

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INTRODUCTION

Most mammals maintain a relatively constant body temperature throughout their adult lives. However, some mammals possess the ability to decrease their metabolic rate and enter a hypometabolic or hibernating state. Such behavior is most often an escape mechanism that extends survival time during challenging environmental conditions (French, 1988).

While in the hibernating state, basal metabolic rates (BMRs) become drastically reduced and body temperatures (T_bs) may drop to a few degrees above the freezing point of water (Lyman, 1982). During this time, nutritional requirements must be met both to maintain cellular activities and to provide energy to rewarm when necessary. Under such conditions and in a fasting state, the hibernator must rely almost entirely upon endogenous foodstores to satisfy these requirements (French, 1988). Examples of facultative metabolic rate depression can be found in both endotherms and ectotherms throughout the animal kingdom (Storey, 1990).

Lipids, which possess a high yield of energy per unit weight, are the primary form of energy storage in hibernating mammals (Aloia and Raison, 1989). In mammals, most lipid is stored as triglyceride in white adipose tissue (WAT) (Aloia and Raison, 1989) and the mass of this tissue decreases steadily throughout the course of hibernation (Lyman, 1982). As such, two critical issues of survival during hibernation are the timing of and the changes in capacity for lipid deposition and utilization (Lyman, 1982).

Stored triglycerides are mobilized from WAT by incorporation into specialized micelles called lipoproteins (Gotto, A.M., Jr., 1987). Lipoproteins are aggregates of neutral lipid and amphiphilic molecules which allow lipids to move freely through the bloodstream. The relative amounts of these lipoprotein components can vary, leading to categorization into three major lipoprotein families: high density lipoproteins (HDLs), low density lipoproteins (LDLs), and very low density lipoproteins (VLDLs).

Previous investigations of lipid metabolism in hibernating mammals have primarily concentrated on the gross plasma levels of metabolites such as triglycerides, with no recognition given to the lipoprotein class from which the lipid originated. Given the unique roles of the individual lipoprotein classes, quantifying the individual lipoprotein classes was the next step to better understand the lipid metabolism of this unique physiological state. This investigation compared the plasma lipoprotein levels of the hibernating *versus* the nonhibernating states in a typical hibernator known as the thirteen-lined ground squirrel, *Spermophilus tridecemlineatus*, using the chromatographic methods of Bentzen *et. al.* (1982). This procedure utilizes affinity chromatography to isolate lipoproteins from plasma samples; these are subsequently quantified enzymatically.

Since the liver is the principal site of lipoprotein synthesis and release, this investigation also estimated the volume density (V_v, volume of specific structure/total volume) of hepatocyte rough endoplasmic reticulum (rER) in the same groups of ground squirrels. This morphometrical analysis was performed to determine whether ultrastructural changes were associated with the hibernating and/or nonhibernating

lipoprotein profiles in a cold environment.

REVIEW OF RELATED LITERATURE

Metabolic Trends During Hibernation

Most of the food consumed by mammals is used for generating heat to maintain their relatively high body temperatures (T_bs). However, the winters of a temperate climate decrease environmental productivity and snow cover frequently prevents foraging for food until spring (French, 1988). In response to these conditions, a group of small mammals which hibernate during the winter rather than expending calories to maintain euthermia has evolved. The metabolic rates of these animals may decrease one hundred-fold while their T_bs can approach 0°C (Lyman, 1982). During hibernation, energy is obtained either from endogenous nutrient reserves or from stockpiles accumulated prior to entering hibernation (Davis, 1976; French, 1988).

A major characteristic of mammalian hibernation is the decreased availability of carbohydrate as an energy source. In 1961, Hannon and Vaughan found that the activity of hepatic glycogen phosphorylase (GP) disappears completely in the hibernating arctic ground squirrel, *Spermophilus undulatus*; this eliminates the liver as a source of glucose. More recently, Storey (1987) examined the activities of GP, phosphofructokinase (PFK), and pyruvate kinase (PK) in the hibernating meadow jumping mouse, *Zapus hudsonis*. He found that the transition to hibernation was accompanied by a dephosphorylation of GP and a phosphorylation of PFK and PK. This caused a significant decrease in the

activities of all three enzymes. Since the modification of GP and PFK occurred within 24 hours of entry into hibernation, the potential for carbohydrate utilization appears to be eliminated quickly in the liver. In a later study, Storey (1989) expanded his findings to include the golden-mantled ground squirrel, *S. lateralis*, which also experienced modified activities of regulatory enzymes upon entering hibernation. Hepatic GP activity was reduced by 50 percent, but PFK and PK activities were unchanged. Additionally, the activity of the mitochondrial pyruvate dehydrogenase (PDH) complex was depressed in all organs by as much as 95% during hibernation. Collectively, these seasonal modifications of enzymatic activity in small rodents restrict carbohydrate availability and utilization during hibernation.

A second major characteristic of hibernation is an increased reliance upon lipid as an alternative energy source. The heavy fat reserves which ground squirrels amass from spring through early autumn are considered to be essential for maintaining homeostasis during dormancy (Aloia and Raison, 1989). Lyman (1982) has noted that the constant decrease in stored fat mass during hibernation is accompanied by a respiratory quotient (RQ) near 0.7, a value indicative of lipid metabolism (for review, see South and House, 1967). In the thirteen-lined ground squirrel, *S. tridecemlineatus*, hibernation has been characterized by elevated levels of triglycerides, free fatty acids (FFAs), and circulating ketone bodies (Galster and Morrison, 1966). Moreover, levels of plasma carnitine and tissue acyl carnitine were elevated significantly relative to a posthibernating euthermic group (Burlington and Shug, 1981). Since carnitine transfers fatty acyl-CoA across the inner mitochondrial membrane, it is considered an essential

component of lipid metabolism (Lehninger, 1982). Increased levels of carnitine have been associated with an increased ability to oxidize free fatty acids (FFAs) (Shug *et. al.*, 1978), which are transported through the plasma in association with albumin. Galster and Morrison (1966) have noted that the plasma levels of albumin increase four-fold during hibernation. The 1975 investigation of Galster and Morrison found that hibernating arctic ground squirrels had depleted levels of liver glycogen, depressed plasma levels of glucose and lactate, and elevated levels of circulating triglyceride.

Introduction to Plasma Lipoproteins

The study of the seasonal dynamics of lipid transport during hibernation involves a specialized micelle called a lipoprotein. Lipoproteins are aggregates of cholesterol, triglyceride, phospholipid, and specialized proteins called apoproteins (also known as apolipoproteins). One characteristic of these "molecules" is that their phospholipid and apoprotein components are amphiphilic. This property is important, since the substrates of lipolysis tend to be sparingly soluble in an aqueous environment. Since quantitative studies show that the amount of neutral core lipid greatly exceeds that of the amphiphilic surface components, it can be deduced that the neutral lipid forms a droplet which is surrounded by a layer of amphiphilic components. In this arrangement, the lipids are held together noncovalently and are organized to reduce the free energy of contact between the neutral lipids and the surrounding aqueous medium (Miller and Small, 1987). The neutral core lipid of lipoproteins consists primarily of triglyceride and cholesteryl esters. The surface layer, which aids in stabilizing the water-lipid interface,

consists of free cholesterol and amphiphilic phospholipids and apoproteins. The relative amounts of these components can vary, resulting in lipoproteins with distinct densities and electrophoretic mobilities. Tables I and II list the composition and physical characteristics of the major human lipoprotein classes.

The classification terms of lipoproteins derived from density-gradient ultracentrifugation with their counterparts from the electrophoretic classifications (Patsch *et.al.*, 1980) should be reconciled. The high density lipoproteins (HDLs), whose floatation densities are greater than $d1.063$, correlate with the lipoproteins that migrate in the "alpha" (α) band during acetate or agarose electrophoresis. Chylomicrons, VLDL, IDL, and LDL, which have floatation densities which are less than $d1.063$ correlate with lipoproteins with an electrophoretic mobility defined as "beta" (β) on acetate or agarose.

To separate the lipoproteins of plasma samples into α (HDL) and β (VLDL + LDL) fractions, the current investigation employed heparin-agarose affinity chromatography (Bentzen, *et. al.*, 1982). This technique correlates well with the separations attained using ultracentrifugation ($r = 0.91$). The chromatographic technique also offers the advantage of readily accommodating the small volumes of plasma obtained from ground squirrels.

Low Density Lipoproteins

Chylomicrons, the largest lipoproteins, are enriched with neutral triglycerides and are released postprandially by enterocytes (Gotto *et. al.*, 1986). The core lipid of chylomicrons is derived principally from dietary fatty acids and 2-monoacylglycerols

TABLE I

CHEMICAL COMPOSITION OF NORMAL HUMAN LIPOPROTEINS[1,2]

	Surface Components			Core Lipids	
	Cholesterol	Phospho-lipids	Apolipo-proteins	Triglycerides	Cholesteryl esters
Chylomicron	35	62	2	95	5
VLDL[3]	43	55	2	76	24
IDL	38	60	2	78	22
LDL	42	58	0.2	19	81
HDL_2	22	75	2	18	82
HDL_3	23	72	2	16	84

[1] From L.C. Smith, J.B. Massey, J.T. Sparrow, A.M. Gotto, Jr., and H.J. Pownall, *in* "Supramolecular Structure and Function", (G. Pifat and J.N. Herak, eds.), p.213. Plenum, N.Y., 1983.
[2] All values are expressed in mol%.
[3] Abbreviations used are as follows: VLDL (very low density lipoprotein), IDL (intermediate density lipoprotein), LDL (low density lipoprotein), and HDL (high density lipoprotein).

(Westergaard and Dietschy, 1976; Johnston, 1977) which become reesterified into triglycerides (also known as triacylglycerols) inside the enterocyte. These triglycerides become packaged into chylomicrons and released into lymphatic ducts (Miller and Small, 1987).

VLDL is also triglyceride-rich, but is synthesized exclusively in the intracellular membrane compartments of hepatocytes; it is released directly into the plasma (Hamilton, 1984). Potential sources of VLDL core triglyceride include free fatty acids obtained from plasma albumin and *de novo* synthesis from precursor units of acetyl-CoA (Sherrill and Dietschy, 1978; Davis *et. al.*, 1979). Similarly, VLDL core cholesterol may be imported into the hepatocyte from the plasma or be synthesized endogenously (Schwartz, 1981). The majority of the synthetic machinery of VLDL formation is located on the

TABLE II

PHYSICAL PROPERTIES OF HUMAN LIPOPROTEIN FAMILIES[1]

	Particle size(nm)	Molecular Weight(D)	Density (g/ml)	Electrophoretic Mobility
Chylomicrons	75-1200	~4,000,000,000	0.93	no migration
VLDL	30-80	10-80,000,000	0.93-1.006	pre-β
IDL	25-35	5-10,000,000	1.006-1.019	slow pre-β
LDL	18-25	2,300,000	1.019-1.063	β
HDL_2	9-12	360,000	1.063-1.125	α
HDL_3	5-9	175,000	1.125-1.210	α

[1] From L.C. Smith, J.B. Massey, J.T. Sparrow, A.M. Gotto, Jr., and H.J. Pownall, in "Supramolecular Structure and Function", (G. Pifat and J.N. Herak, eds.), p.213. Plenum, N.Y., 1983.

cytoplasmic side of the ER membranes of hepatocytes (Bell, *et. al.*, 1981).

Once released into the plasma, further metabolism of chylomicrons and VLDL depends on three apoproteins located on the surface of the particle: apoprotein (apo)CII, apoE, and apoB100. The initial catabolic step involves the loss of virtually all core triglycerides (see Figs. 1-3). This process of delipidation is catalyzed by the enzyme lipoprotein lipase (LPL). There is general agreement that LPL is synthesized in paranchymal cells, secreted, and bound to capillary endothelial cells by glycosaminoglycans (Nilsson-Ehle *et. al.*, 1981, Borensztajn, J., 1987). ApoCII is a cofactor in the LPL catalyzed reaction and is transferred to mature, circulating chylomicrons and VLDL from circulating HDL (Nikkila, 1983; Smith and Pownall, 1984). Interaction of LPL with apoCII and the core triglyceride of the lipoprotein hydrolyzes triglyceride (and some phospholipid), while simultaneously decreasing the

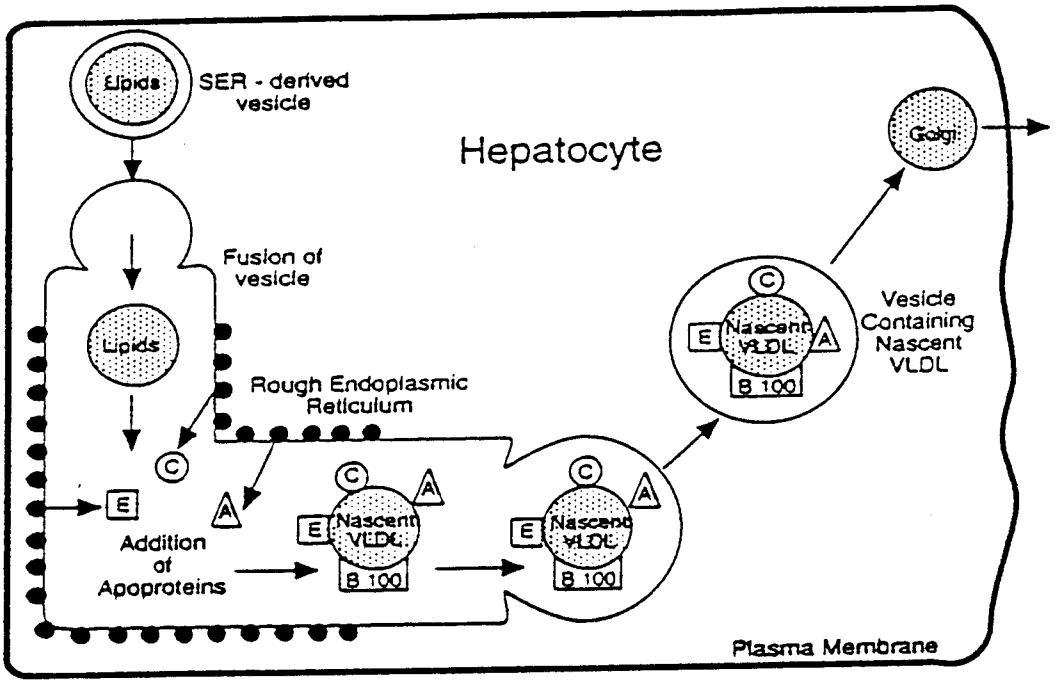

Figure 1. Overview of the assembly of VLDL within the hepatocyte. Lipid particles (cholesterol, triglyceride, and phospholipid) derived from vesicles of the sER become sequestered into the lumen of the rough endoplasmic reticulum. Apoproteins are added to the surface of the particle and the nascent VLDL is packaged into a vesicle.

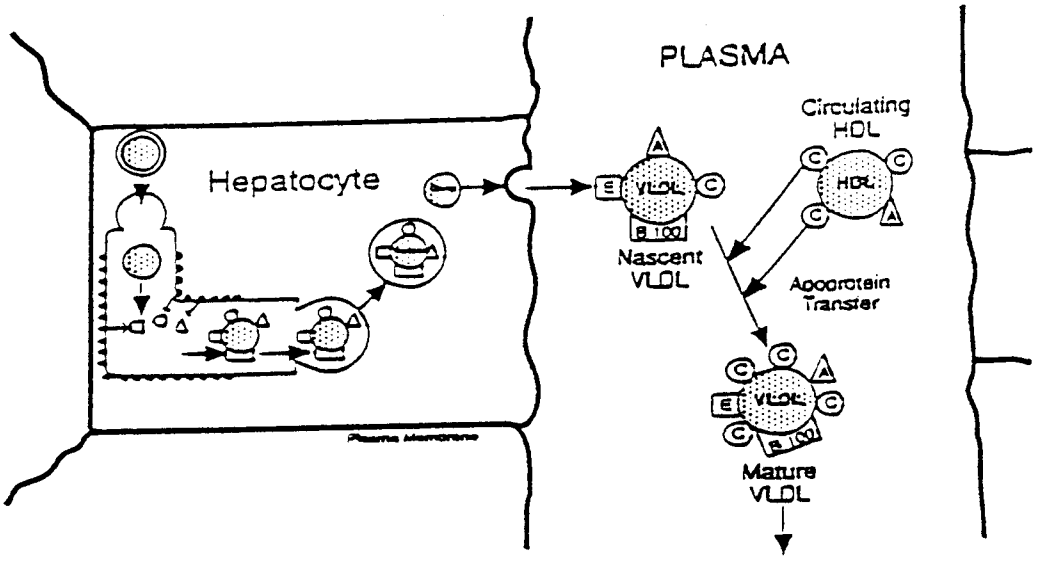

Figure 2. Illustration of plasma modification of nascent VLDL. Once released into the plasma, nascent VLDL matures through contact with circulating HDL. These contacts result in HDL lipoproteins being transferred to the nascent VLDL. After accepting these apoproteins, the VLDL is considered mature.

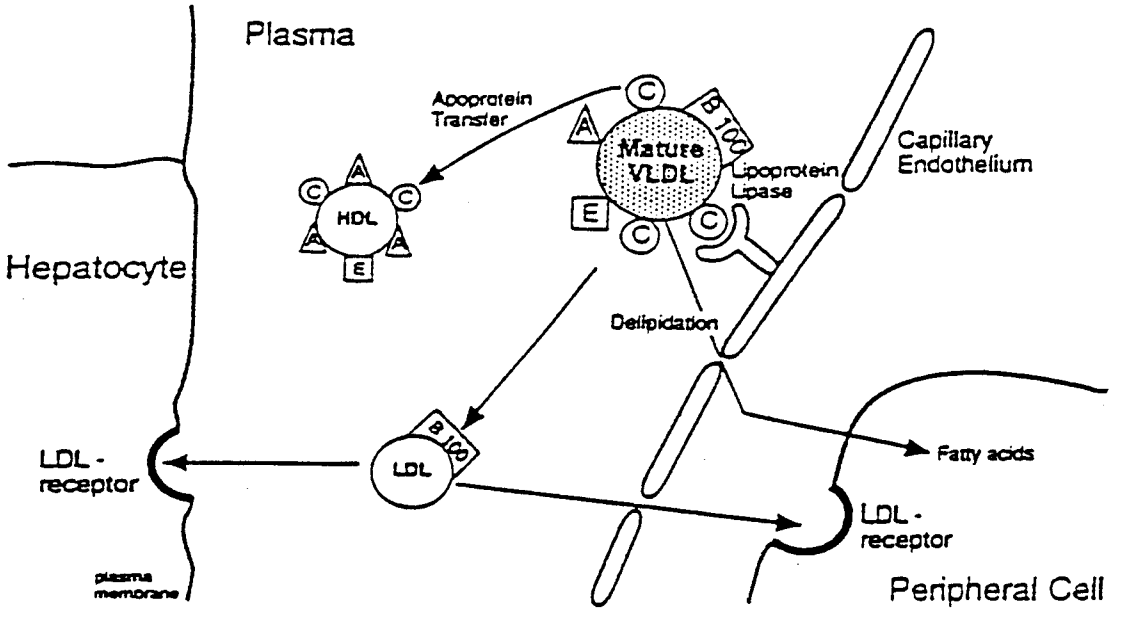

Figure 3. Overview of the catabolism of VLDL and the formation of LDL. VLDL catabolism is initiated by the interaction of apoprotein C with the enzyme lipoprotein lipase (LPL). This enzyme is located on the plasma side of the capillary endothelium. Following delipidation and the loss of all apoproteins except B100, the VLDL remnants become organized into LDL. LDL is cleared from the plasma *via* interaction with LDL receptors.

mass of the lipoprotein. While core mass is decreasing, apoCII is released from the surface of the lipoprotein and returns to circulating to HDL (Havel, 1978).

After undergoing delipidation, chylomicrons and large VLDLs are thought to retain both apoE and apoB100. The remnants of this process become organized into lipoproteins known as chylomicron and VLDL remnants; the latter also are referred to as IDL. Virtually all chylomicron and VLDL remnants are internalized by hepatocytes via the interaction of apoE with hepatic remnant receptors (Bradley *et. al.*, 1984; Havel *et. al.*, 1984).

In contrast, smaller VLDLs are thought to lose their apoE components during delipidation and become organized into LDL (Reardon *et al.*, 1978). Unlike VLDL remnants, LDLs possess an apoprotein profile that is almost entirely apoB100 (Fisher, 1983). LDL catabolism is initiated by the interaction of apoB100 with an LDL receptor found on both hepatocytes and extrahepatic cells. To date, LDL receptors have been found on the surface of virtually every cell type tested, though their numbers tend to be lower on nonmitotic cells which require less cholesterol (Gianturco and Bradley, 1987). The binding of LDL and its subsequent lysosomal hydrolysis suppresses further expression of the LDL receptor. It also inhibits the activity of HMG CoA reductase (3-hydroxy-3-methylglutaryl reductase), which limits endogenous cholesterol synthesis (for review, see Brown and Goldstein, 1986). Thus, by means of chylomicrons, VLDL and LDL, triglyceride-rich lipoproteins provide a pathway by which the liver and small intestine can provide triglyceride and cholesterol for peripheral cellular metabolism.

High Density Lipoproteins

HDLs originate as nascent, discoidal precursors composed mainly of surface components and apoAI and/or apoE. Precursors may originate in several ways: (1) by direct secretion from the liver or small intestine as secretory nascent HDL (Gotto *et. al.* 1986) (2) from the remnants of hydrolyzed triglyceride-rich lipoproteins (Patsch *et. al.*, 1978), and (3) by complex interaction of apoproteins and phospholipids circulating in the plasma (Pownall *et. al.*, 1982).

Once circulating, the discoidal precursor attains a spherical shape by accumulating a core of cholesteryl ester. This transition is catalyzed by the enzyme lecithin/cholesterol acyl transferase (LCAT), which requires apoAI as a cofactor (Schaefer *et. al.*, 1982; Norum, *et. al.*, 1982). In this manner, free cholesterol which is encountered by the nascent particle becomes incorporated onto its surface. This newly acquired surface cholesterol can then be esterified by LCAT and migrate to the core of the molecule, splitting the surface components until a spherical HDL is formed (Patsch and Gotto, 1987).

Mature, spherical HDLs are subject not only to the dynamic equilibria of the plasma, but also to interaction with other lipoprotein classes and lipid sources. The exchange of cholesterol, cholesteryl ester, triglyceride, and apoproteins represents the most common method of HDL modification. For example, six to eight hours after ingestion of a fat-rich meal, HDLs become enriched with triglyceride. The extent of the enrichment is proportional to the degree of postprandial lipemia and to the rate of triglyceride-rich lipoprotein catabolism (Patsch *et. al.*, 1984).

A common method of lipid transfer involves spontaneous dissociation of lipid from a donor to an acceptor. Lipid from plasma membranes can spontaneously dissociate into the plasma and become rapidly incorporated into a circulating lipoprotein or other lipid acceptor (Smith and Gotto, 1985). This type of spontaneous transfer is limited by the exceedingly low solubility of lipophilic material in an aqueous environment. The apoprotein components of HDL may also exchange with other lipoprotein classes. For example, the C class of apoproteins can readily exchange between HDL and the triglyceride-rich lipoproteins (Eisenberg and Levy, 1975; Schaefer *et. al.*, 1978), its distribution at any time is governed by the relative concentrations of the two lipoprotein types. As an example of this type of equilibrium, during alimentary lipemia the plasma concentration of chylomicrons becomes greatly increased. During this time, the majority of the C apoproteins are transferred from HDL to the chylomicrons. Upon the delipidation of these chylomicrons, the C apoproteins return to HDL (Havel *et.al.*, 1973).

There are two major subclasses of HDL: HDL_2 and HDL_3. Currently, it is unknown whether maturation of nascent HDL leads to the formation of HDL_2, HDL_3, or both (Patsch and Gotto, 1987). In 1978, Patsch proposed a mechanism by which the action of LPL may cause the conversion of HDL_3 to HDL_2. Initially, triglyceride-rich lipoproteins would undergo lipolysis and liberate lipid components which are transferred to HDL_3. As this enrichment continues, the density of HDL_3 would decrease to that of HDL_2. In addition to the LPL catalyzed transfer, LCAT must be involved to allow these lipids to enter the core of the HDL (Patsch *et. al.*, 1977; Schmitz *et. al.*, 1981; Darr and

Greten, 1982). Although this complete model has not been demonstrated *in vivo*, components released during lipolysis have been shown to be incorporated into existing HDL_3s. Experiments have also demonstrated that HDL_2 may be converted back to HDL_3 by the action of hepatic lipase, which facilitates the exchange of HDL_2 cholesterol esters for triglycerides. Hepatic lipase, which does not require apoCII as a cofactor, then hydrolyses the triglycerides of HDL_2, thereby reducing the core mass back to that of HDL_3 (Patsch *et. al.*, 1984).

Though not included in Tables I or II, a third class of HDL exists called HDL_1. Though a minor species in humans, it is a major lipoprotein in the rat (Lusk, *et. al.*, 1979; Patsch et. al., 1980; Oschry and Eisenberg, 1982). HDL_1 has a diameter of 12-14nm, and is so enriched in lipid (mainly cholesterol) that its density resembles that of LDL. Its apoproteins are mainly AI and E (it is deficient in AII). Due to the presence of apoE, HDL_1 may interact with the chylomicron receptor (which recognizes apoE) and/or the LDL receptor. It would be catabolized in a manner similar to that of a chylomicron remnant or large VLDL.

While specific HDL receptor proteins have not yet been purified, studies have shown that binding is potentially regulated by apoproteins AI and AII (Fidge and Nestel, 1985). Binding, internalization, and degradation of apoE free HDL have been demonstrated in the liver, kidney, intestine, adipocytes, fibroblasts, and adrenal cortical cells (Eisenberg, 1984). The final catabolism of HDL particles may consist of two distinct pathways. This is suggested by the work of Glass *et. al.* (1983) and Lietersdorf *et.al.* (1984), who have shown that the uptake of HDL cholesterol by hepatocytes is 3-8

times greater than is the uptake of HDL apoproteins. Also, the apoproteins of HDL need not become degraded during the process of cholesterol donation or acceptance. It has been shown that HDL binding is not necessarily followed with internalization, and that internalization is not necessarily followed by degradation (Kagami *et. al.*, 1984; Pittman and Steinberg, 1984). When internalized but not degraded, the particle is extruded from the cell in a process known as retroendocytosis.

Pittman and Steinberg (1984) have studied HDL catabolism in the rat and determined that the greatest uptake/unit tissue occurs in the adrenals and gonads. However, at least six times as much cholesterol ester as protein was removed by the adrenals. The liver was quantitatively the major site of HDL removal, removing one-third of the total HDL protein and two-thirds of the total cholesterol. The kidney was a major site for apoprotein removal and degradation (Glass *et. al.*, 1983). The net result of these findings can be summed by stating that HDL lipids and proteins may ultimately be catabolized in different locations. Thus, HDL is considered to be a dynamic molecule which can function in a number of capacities. It can accept and distribute lipid and apoproteins, thereby stabilizing the products of lipolytic degradation. It also has a critical role as a "scavenger" of free or excess lipid, which it returns to the liver for excretion.

Mammalian Lipoprotein Profiles

Extensive homology has been demonstrated between human and other mammalian lipoproteins (Tables III-V) (Chapman, 1980 and 1986). For example, chylomicrons from

TABLE III

COMPARISON OF PHYSICAL PROPERTIES OF MAMMALIAN VLDL

Species	Property				
	Density Interval (g/ml)	Particle Diameter Å		Hydrated Density (g/ml)	Electrophoretic Mobility
		Mean	Range		
Human[a]	<1.006	364	250-500	-	pre-β
Mouse[b]	<1.017	494	270-750	0.971	VLDL
Rat[c]	<1.006	570	200-900	0.965	pre-β
Guinea Pig[d]	<1.006	463	306-775	0.969	pre-β
Chimpanzee[e]	<1.007	417	250-600	0.995	VLDL

[a] Normolipidemic, fasting subjects; J.P. Kane, T. Sata, R.L. Hamilton, and R.J. Havel, *J. Clin. Invest.* **56,** 1622, (1975)

[b] M.C. Camus, M.J. Chapman, P. Forgez, and P.M. Laplaud, *J. Lipid Res.* **24,** 1210, (1983).

[c] O.D. Mjϕs, O. Faergeman, R.L. Hamilton, and R.J. Havel, *J. Clin. Invest.* **56,** 603, (1975)

[d] M.J. Chapman and G.L. Mills, *Biochem J.* **167,** 9, 1977.

[e] M.J. Chapman, P. Forgez, D. Lagrange, S. Goldstein, and G.L. Mills, *Atherosclerosis,* **52,** 129, (1984).

TABLE IV

COMPARISON OF PHYSICAL PROPERTIES OF MAMMALIAN LDL

Species	Property				
	Density Interval (g/ml)	Particle Diameter Å		Hydrated Density (g/ml)	Electrophoretic Mobility
		Mean	Range		
Human[a]	1.030-1.040	247	-	-	β
Mouse[b]	1.023-1.060	244	220-280	1.034	LDL/β
Rat[c]	-	280	-	1.031	β
Guinea Pig[d]	1.030-1.100	214	125-280	0.969	β
Chimpanzee[e]	1.024-1.050	220	160-280	1.053	β

[a] D.M. Lee, and P. Alaupovic, *Biochemistry,* **9,** 2244, (1970).
[b] M.C. Camus, M.J. Chapman, P. Forgez, and P.M. Laplaud, *J. Lipid Res.* **24,**1210, (1983).
[c] L.T. Lusk, L.F. Walker, L.H. DuBien, and G.S. Getz, *Biochem. J.* **183,** 83, (1979).
[d] G.L. Mills, M.J. Chapman, and F. McTaggert, *Biochim. Biophys. Acta* **260,** 401, 1972.
[e] M.J. Chapman, P. Forgez, D. Lagrange, S. Goldstein, and G.L. Mills, *Atherosclerosis,* **52,** 129, (1984).

TABLE V

COMPARISON OF PHYSICAL PROPERTIES OF MAMMALIAN HDL

Species	Property: Density Interval (g/ml)	Particle Diameter Å Mean	Particle Diameter Å Range	Property: Hydrated Density (g/ml)	Property: Electrophoretic Mobility
Human[a]					
HDL2	1.063-1.125	100	-	1.105	-
HDL3	1.125-1.210	75	-	1.153	-
Mouse[b]	1.063-1.210	~131	-	1.154	-
Rat					
HDL1[c]	-	127	89-178	1.072	-
HDL2	1.085-1.210	92	70-118	1.124	-
HDL3[d]	1.125-1.210	~55	-	-	-
Guinea Pig[e]	1.090-1.210	97	-	1.115	α
Chimpanzee	-	-	-	-	-

[a].M. Scanu, L. Vitelo, and S. Deganello, *CRC Rev. Biochem.* **2,** 175, (1974).
[b] M.C. Camus, M.J. Chapman, P. Forgez, and P.M. Laplaud, *J. Lipid Res.* **24,**1210, 1983.
[c] Y. Oschry and S. Eisenberg, *J. Lipid Res.* **23,** 1099, (1982).
[d] I. Pasquali-Ronchetti, S. Calandra, and M. Montaguti, *J. Ultrastruct. Res.* **53,**180, (1975).
[e] C. Sardet, H. Hansma, and R. Ostwald, *J. Lipid Res.* **13,** 180, (1975).

all mammalian species typically consist of 1-2% protein (by weight) and 98-99% lipid. Of the lipid, 90% is usually triglyceride, 5-8% is phospholipid, 1-2% is cholesteryl ester, and 1% is cholesterol. VLDLs contain a higher percentage of protein (~7-10%) and normally have a lipid profile of 65% triglyceride, 12% cholesterol ester, 18% phospholipid, and 5% cholesterol (Miller and Small, 1987). As a result of these similarities, lipoproteins from different mammalian species can generally be efficiently separated using procedures based upon physical characteristics or protein binding specificities. As examples, the density boundaries which define the limits of the human lipoprotein classes are routinely applied to other mammalian species, and procedures such as heparin-agarose affinity chromatography have been used to isolate lipoprotein classes from mammalian species such as the human and the rat. As such, the preceding information concerning lipoproteins has been compiled from both human and rat data, but is equally applicable to the lipoproteins of other mammalian species.

The present investigation was designed to examine lipoprotein traffic in the hibernating ground squirrel, *S. tridecemlineatus*. Previous studies of lipid metabolism in hibernators have often yielded seemingly contradictory data. Although the reasons for this are unclear, some answers may lie in the length of time required for hibernation-induced alterations to become evident. For example, after allowing arctic ground squirrels several months to acclimate to cold room conditions, Galster and Morrison (1975) found that total plasma triglycerides increased during hibernation. Conversely, using an acclimation period of eight days, Nizielski *et. al.* (1989) found that circulating triglyceride levels in the thirteen-lined ground squirrel were reduced. To minimize such

problems, the present investigation used an extended acclimation period of 14 weeks to distinguish an initial, transitional metabolism from a long term capacitive adaptation to the cold.

Additionally, the majority of studies of hibernating lipid metabolites have focused on total plasma levels of circulating lipid metabolites. Using such methods, all plasma lipids have been reported as a single sum, with no recognition of the different physiological roles of the lipoprotein classes in which they resided. The logical next step was to quantify the circulating plasma lipids with respect to their residence in the plasma lipoprotein classes.

To accomplish this end, micro-scale heparin-agarose affinity chromatography was used to separate the lipoprotein classes of plasma samples (Bentzen, C.L. et. al., 1982). This technique is based on the affinity between certain plasma lipoproteins and a class of molecules called glycosaminoglycans (Iverius, 1972). By binding various glycosaminoglycans to agarose beads, heparin was found to yield the best separation between HDL (α) and VLDL+LDL (β) (Iverius, 1972). The selective binding of the lipoprotein classes is due to the interaction of heparin with apoproteins B100 and E. These apoproteins are present on the classes VLDL and LDL, but not on the HDL. When eluting a plasma sample through a heparin-agarose column with a near physiological strength saline eluent, the β lipoproteins of the plasma sample remain tightly bound to the column. The α lipoproteins, which do not interact with heparin, elute through the column. Higher ionic strengths of eluent can be subsequently employed to desorb and elute the β lipoproteins (Iverius, 1972; Isolab Technical Bulletin, 1984).

After separation, the lipoproteins may be quantified enzymatically to determine the amount of cholesterol in each fraction.

In addition to quantitating the levels of α and β lipoproteins, this investigation also compared the ratios of these lipoproteins from each group to each other. Ratios of this type have their origin in the research of Miller and Miller (1975), who proposed that α lipoproteins may protect against the development of coronary artery disease (CAD) in humans. The negative association of α lipoproteins and CAD has since been verified (Gordon *et.al.*, 1977; Miller, 1981).

Bentzen *et. al.* (1982) proposed that the ratio of the plasma lipoproteins to each would be a more reliable predictor of CAD risk than would the individual plasma levels of α and β lipoproteins. These are qualitative values and do not reflect the quantity of lipoproteins responsible for the ratio. Since these ratios were determined to be sensitive predictors of physiological conditions such as CAD in humans (Bentzen, 1982), it might be hypothesized that the ratio of the α and β lipoproteins to each other may be as important as their individual plasma levels when describing the state of lipid metabolism in a mammal.

A second component of this investigation used transmission electron microscopy (TEM) and standard morphometrical techniques to analyze the hepatocytes of the warm and the cold adapted ground squirrels. Since hepatocytes are the major site of lipoprotein synthesis (Gotto, A.M., 1987), this morphometrical investigation was intended to determine whether quantitative changes of rER were associated with any potentially altered lipoprotein profiles due to cold exposure and/or hibernation. The validity of such

morphological techniques was shown by Staubli *et.al.* (1969), who catalogued quantitative changes in the rER of rat hepatocytes in response to treatment with phenobarbital. This established the basis for a quantitative correlation of liver structure with cell function as determined by biochemical methods (Weibel *et. al.*, 1969; Staubli *et. al.*, 1969).

The ability of hibernating mammals to synthesize proteins has been documented. Whitten and Klain (1968) found that the incorporation of ^{14}C-labeled methionine into the protein of liver slices of *S. tridecemlineatus* was reduced by about 2/3 in both hibernating squirrels and squirrels in the process of awakening from hibernation. The control group in this study consisted of spring posthibernating ground squirrels. In two subsequent studies, Whitten *et. al.* (1970 a,b) reported that hepatic polyribosome levels and rates of protein synthesis of *S. tridecemlineatus* were greatly diminished in winter hibernating and winter euthermic squirrels relative to summer euthermic ground squirrels. Since decreases were found in both winter euthermic and hibernating squirrels, this could indicate that the variation from the control values may represent a seasonally induced change rather than an adaptation to the hibernating state.

Along with these cold and/or hibernation-associated biochemical changes, many authors have documented ultrastructural variations associated with cold exposure and hibernation. Toth (1980) found that the cytoplasm of Paneth cells from hibernating *S. lateralis* were characterized by poorly organized rER, decreased numbers of ribosomes/cell, and an increased percentage of unbound and unaggregated ribosomes. The nuclei of hibernating cells were characterized by diminished nuclear size, a less

prominent nucleolus, and increased amounts of heterochromatin.

More recently, Steffen *et. al.* (1991) have noted differences between the densities of fibres and capillary beds of the soleus and extensor digitorum longus muscles in summer active and hibernating *S. lateralis*. Additional morphological changes associated with hibernation have been reported on thyroid tissue from *S. richardsoni* by Winston and Henderson (1980), on juxtaglomerular cells from *S. tridecemlineatus* by Zimny *et. al.* (1984), and on cardiac tissue from *S. tridecemlineatus* and *S. undulatus* by Darvish and Black (1986) and by Zhegunov (1988), respectively. In all of these investigations, comparisons were made between hibernators and a control group consisting of posthibernating animals.

Ultrastructural modifications associated with cold adaptation have also been addressed in nonhibernating mammals. Desautels and Himms-Hagen (1980) found that cold exposed Charles River Holtzman rats, which employ BAT as a cold-resistive adaptation, exhibited decreases in BAT mass during the first week of cold exposure before returning to control values. It was also found that the mitochondria from the cold exposed group possessed larger than normal dimensions, condensed matrices, and an enlarged intracristal space. Additionally, Pollera *et. al.* (1983) have shown that peroxisomes of the hepatocytes of Sprague Dawley rats were increased in number and volume after seven days of cold exposure (5°C).

In light of this information, this investigation will determine if there exists a correlation between lipoprotein data obtained from the plasma of the ground squirrels and the morphological data of their hepatocytes. In doing so, a more complete picture may

evolve regarding lipid metabolism during cold adaptation and/or hibernation.

METHODS AND MATERIALS

Acclimation History of Ground Squirrels

Thirteen-lined ground squirrels (*S. tridecemlineatus*) were captured in northern Illinois by TLS Research (Chicago, Illinois) and delivered to the Animal Care Facility of Loyola University of Chicago. Squirrels were housed individually in shoebox cages with dimensions of 45x24x22 cm. All animals were provided with corncob bedding and nonabsorbent cotton, the latter serving as nesting material to minimize captivity stress. The temperature of the room was maintained at 19°C with a photoperiod of 12:12 hr (light:dark). Throughout the course of this investigation water and Purina Rodent Chow were supplied *ad libitum*, supplemented periodically with apples and sunflower seeds.

Beginning in late November, 1990, ground squirrels were acclimated for 14 weeks to either a warm or cold environment. The control group, designated Group I, consisted of seven squirrels which were maintained under the previously described conditions. Animals in this group had T_bs of 25°C or higher when measured rectally with an Omega^R HH81 digital thermometer.

Cold adapted animals were subdivided into two groups. Group II consisted of six squirrels which were hibernating in a darkened Hotpak^R environmental chamber maintained at 4°C. These conditions were intended to simulate burrow conditions. Prior to sample collection, animals which assumed the "curled up" posture characteristic of

hibernation had their food replaced with a small quantity of sunflower seeds. If squirrels appeared dormant and had not disturbed the seeds for 14 hours, they were considered to be hibernating. This observation was reinforced if their rectal temperatures were 10°C or lower at the time of sampling. Group III, hereafter referred to as "arousals", consisted of six squirrels maintained in cold which had awakened from the hibernating state. The squirrels were alert, active, and maintaining T_bs over 30°C. Periodic arousal from the dormant state is considered to be a natural part of hibernation in small mammals (Galster and Morrison, 1975; Lyman, 1982).

Blood Collection

Sampling was carried out between late February and mid-March, 1991. In order to obtain fasting blood samples, food pellets were withdrawn from the control and arousal groups 14 hours prior to blood collection. As mentioned previously, the food of the hibernating squirrels was replaced with sunflower seeds 14 hours prior to sampling to determine whether the squirrels remained dormant. Untouched sunflower seeds indicated that the squirrel had not eaten during this period.

Squirrels were decapitated and trunk blood was collected directly into one ml of anticoagulant (0.9% NaCl, 0.1% EDTA) which had been chilled by storage on ice. Chilled plasma was separated from cellular components by centrifugation at 1,000g for 10 minutes in an IEC tabletop centrifuge. Plasma was then aspirated from the centrifugation tubes and either used immediately or stored for up to three days in sealed

containers at 4°C. Refrigerated samples of plasma have been demonstrated to be stable for this time period (Isolab Technical Bulletin, 1984).

Affinity Chromatography

Separation of plasma lipoproteins was accomplished according to the modified technique of Bentzen *et. al.* (1982). Micro-affinity columns packed with heparin-agarose were obtained from Isolab Inc., Akron, Ohio. Column diameters were 0.325cm and bed heights were approximately 3cm, resulting in bed volumes of approximately 0.25ml. The heparin-agarose medium of the columns was overlaid with an application filter with an average pore size of 30 μm.

Prior to sample application, each column was equilibrated with 2ml of 0.11M NaCl. The column was then positioned over a collecting tube and 0.2ml of plasma sample was loaded onto the column. The eluant which was collected constituted the first 0.2ml of the alpha (α) fraction. Next, 1.0ml of 0.11M NaCl was applied to the column and the second eluant collected into the same tube. The second eluant contained the α lipoproteins (HDLs), and the combined volume of the eluants raised the final volume of the α fraction to 1.2ml.

The beta (β) lipoproteins (VLDL, LDL) were eluted from the column by the addition of 1.2ml of 1.0M NaCl. This eluant was collected into a second collecting tube and constituted the β fraction.

To insure that no cross-contamination of the α and β fractions would occur, trials

were performed in which the α fraction was eluted several times with 0.11M NaCl. These trials revealed that the β fraction remained bound to the column when the eluant intended to elute only the α fraction was employed. Additionally, repeated elutions with 1.0M NaCl indicated that virtually no lipoproteins remained residually bound to the column following the initial elutions with 0.11M and 1.0M NaCl.

Fraction Analysis

The α fraction, the β fraction, and the diluted plasma sample were each assayed enzymatically to determine their cholesterol content. Quantitation was performed using Sigma Diagnostics Cholesterol Procedure No. 352 (Sigma Chemical Co., St. Louis, Mo.). The reconstituted Sigma reagent contained the following final concentrations of components: cholesterol oxidase (microbial), 300 U/L; cholesterol esterase (microbial), $>$ 100 U/L; peroxidase (horseradish), 1000 U/L; 0.3 mM 4-aminoantipyrine; 30.0 mM p-hydroxybezenesulfonate; and phosphate buffer, pH 6.5.

Prior to combining the cholesterol reagent and the samples in test tubes, the reagent was preheated to 37°C in a circulating water bath. After warming, 0.1 ml of cholesterol standard, α fraction, β, or diluted plasma was added to 0.9ml of cholesterol reagent. The reactants were gently mixed and allowed to react for 15 min in a 37°C water bath.

The reaction sequence was as follows:

$$\text{Cholesterol Esters} + H_2O \xrightarrow{CE^*} \text{Cholesterol} + \text{Fatty Acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{CO} \text{Cholest-4-en-3-one} + H_2O_2$$

$$2H_2O_2 + 4\text{-AAP} + \text{p-HBS} \xrightarrow{HP} \text{Quinoneimine Dye} + 4H_2O$$

*Key: CE = cholesterol esterase
CO = cholesterol oxidase
4-AAP = 4-aminoantipyrine
p-HBS = p-hydroxybenzenesulfonate
HP = horseradish peroxidase

The quinoneimine dye produced by this reaction sequence has an absorption peak at 500nm. Absorbance readings were made after removing test tubes from the water bath and allowing the reaction mixture to stand for 10 min at room temperature. The intensity of the color produced by the reaction is directly proportional to the cholesterol concentration of the sample. All readings were made at 500nm with a Beckman DU-64 Spectrophotometer. Cholesterol concentrations were estimated by dividing the absorbance of the sample by the absorbance of the 250 mg/dl cholesterol standard. The quotient was then multiplied by 250, the cholesterol concentration of the standard. This yields a concentration value in units of mg/dl. The ratio of β cholesterol to total cholesterol in each squirrel was determined by dividing the cholesterol concentration of its β fraction by the cholesterol concentration of its diluted plasma sample.

Calculations were performed as follows:

$$[Cholesterol]_{sample} = \frac{A_{500}\ sample}{A_{500}\ standard} \times [Cholesterol]_{standard}$$

$$\%Recovery = \frac{\alpha\ cholesterol + \beta\ cholesterol}{plasma\ cholesterol} \times 100$$

$$Percent\ \beta\ (\%\beta) = \frac{\beta\ cholesterol}{total\ cholesterol}$$

A standard curve of cholesterol concentrations was established through the use of cholesterol standards (Sigma Chemical Co., St. Louis) of 250, 200, 150, 100, and 50 mg/dl concentrations.

To determine the efficiency of the columns, the total cholesterol concentration of the plasma sample was compared with the sum of the cholesterol concentrations of the α and β fractions. Since the process of separation diluted the fractions by a factor of six, the plasma samples were diluted 1:6 with 0.15M NaCl prior to being assayed. Average percent recovery was calculated to be 99.0 $\pm$ 0.88 (S.E.M.).

Preparation of Tissue For Electron Microscopy

Immediately after blood collection, hepatic tissue was excised from the right medial lobe of each squirrel and immersed in fixative to preserve its ultrastructure. Tissue samples of roughly 5 mm^3 were immersed into 2 ml of cold (4°C) Karnovsky's media, which consisted of 25ml 8% p-formaldehyde, 50ml 0.2M cacodylate buffer, 10ml 10% glutaraldehyde, and 15ml distilled, deionized (dd) H_2O. The tissue was then razor minced into pieces no larger than $1mm^3$. The tissue samples were stored in cold (4°C) Karnovsky's fixative until subsequent procedures were performed.

The tissue was then washed five minutes in cold 0.2M cacodylate buffer (42.8g sodium cacodylate [$Na(CH_3)2AsO_2$ $3H_2O$], dd H_2O to one liter, 10ml concentrated [36-38%] HCl, 603ml distilled water). This procedure was repeated twice. Following the final wash, tissue was post-fixed in 2% osmium tetroxide at room temperature for

one hour. The post-fixed tissue was washed in cold cacodylate buffer, and dehydrated at room temperature through a graded acetone series. Dehydration was performed by immersing tissue for five minute periods in increasing concentrations of acetone (once each in 30%, 50%, 75%, and 90% acetone, and three times in 100% acetone).

Dehydrated tissue was embedded in a 1:1 acetone:Epon 812 resin mixture (29g DDSA, 27g NMA, 52.3g Epon 812, DMP-30 @ 1.5ml/100ml resin) for four hours at room temperature. The tissue was then placed into a 1:3 acetone:resin mixture at room temperature. The next day, tissue was transferred into 100% resin and placed into an evacuated chamber for approximately ten hours. In the final step, the tissue was placed into molds containing 100% resin and allowed to polymerize in an oven for four days at 60°C.

Tissue was thin-sectioned using an RMC MT-5000 microtome and 80 nm sections collected on 300 mesh copper grids. Sections were stained for five minutes with 4.7 x 10^{-2}M uranyl acetate, washed in dd H_2O, then stained with lead citrate (3.01 x 10^{-2}M lead nitrate, 2.63 x 10^{-2}M lead acetate, 9.48 x 10^{-3}M lead citrate, 3.40 x 10^{-2}M sodium citrate) for one minute. Tissue was washed and allowed to dry for at least several hours before being viewed and photographed with a JEOL-1200 EX transmission electron microscope.

Morphometrics

The morphometrical sampling procedure for this investigation was based on the methods of Weibel (1979). The modified sampling procedure randomly selected three ground squirrels from each of the three groups (controls, hibernators and arousals).

From each of the nine selected squirrels, three randomly selected blocks of tissue were thin sectioned. One section from each block was photographed four times at random locations at a magnification of 12,000x, giving a total of 108 negatives. Each negative was printed at the enlarged magnification of 27,000x. The volume density (V_v) of rER was calculated by overlaying each of the 108 photographs with a square grid possessing 550 points of intersection (Thomson, E., 1930; Glogalev, A.A, 1933). The number of times that the rER contacted a point of intersection was counted and recorded as a percentage of 550. The mean percentage values were expressed $\pm$ S.E.M.

Statistics

Analysis of lipoprotein and morphometrical data was performed with a single factor, two-tailed analysis of variance (ANOVA) using the statistical models found of Zar (1984). An ANOVA yielding a significant difference ($P < 0.05$) was followed by the multiple comparison test of Tukey, which allowed specific intergroup comparisons to be examined.

RESULTS

Lipoprotein Analysis

Table VI lists the results of the quantitation procedure for lipoprotein cholesterol and the ratios of β/total cholesterol obtained from the control, hibernating and arousal groups. Relative to the control group, the hibernating and arousal groups showed increased α and decreased β levels of lipoprotein cholesterol. The α lipoprotein cholesterol was lowest in the control group (184mg/dl), higher in the hibernators (210mg/dl), and highest in the arousals (219mg/dl). The β lipoprotein cholesterol was highest in the control group (96mg/dl) and lower in the hibernators (80.9mg/dl) and the arousals (62.4mg/dl). The total of lipoprotein cholesterol (α plus β) remained relatively constant among the three groups.

As determined by a one factor, two-tailed analysis of variance (ANOVA), the concentrations were not significantly different among the total, α, or β lipoprotein cholesterol fractions (Table VII). However, when the ratio of β lipoprotein cholesterol was considered as percentage of total lipoprotein cholesterol, a significant difference existed among the three groups. To determine which comparison caused the significance, the multiple comparison method of Tukey was employed (Table VIII). In this test, groups were ranked in descending order by the magnitude of their mean differences. Once an insignificant result was obtained, no further comparisons were warranted. The

Figure 4: Micrograph of ground squirrel hepatocyte.

Representative micrograph of an hepatocyte from the ground squirrel, *Spermophilus tridecemlineatus*. Mitochondria (M) and rough endoplasmic reticulum (rER) are located amid abundant glycogen reserves (G). A nucleus (N) is also present. Scale bar = 1 μm.


G
rER
M
N

results indicated that the arousal group had a significantly lower β/total cholesterol ratio than the control group ($P = 0.004$). No significant differences were found in comparisons of control *vs.* hibernator and arousal *vs.* hibernator.

TABLE VI

PLASMA LIPOPROTEIN CHOLESTEROL[1]

	Control (n=7)	Hibernator (6)	Arousal (6)
α(HDL) cholesterol	184 ± 13.6[2]	210 ± 23.5	219 ± 20.6
β (VLDL+LDL) cholesterol	96.0 ± 13.4	80.9 ± 8.42	62.4 ± 6.49
Total cholesterol	272 ± 26.0	296 ± 27.9	288 ± 25.1
β/total (arcsin)[3]	0.346 ± 0.023	0.277 ± 0.023	0.219 ± 0.022

[1] Cholesterol values expressed in mg/dl.
[2] Mean value ± S.E.M.
[3] Arcsine transformation for small or large percentages (Zar, J.H., 1984).

TABLE VII

ANOVA OF PLASMA LIPOPROTEIN CONCENTRATIONS

	F-RATIO	Probability	Result
α (HDL) cholesterol	0.971	0.400	NS
β (VLDL + LDL) cholesterol	2.792	0.091	NS
Total cholesterol	0.227	0.800	NS
β/Total	7.879	0.004	$P = 0.004$

TABLE VIII

TUKEY TEST OF β/TOTAL CHOLESTEROL

Comparison (A *vs.* B)	Difference (X_A-X_B)	SE	q	$q_{(0/05,16,3)}$	Result
control vs. arousal	0.127	0.0215	5.90	3.649	P=0.004
control vs.hibernator	0.069	0.0215	3.21	3.649	NS
arousal vs. hibernator			NOT TESTED		

Morphometrical Analysis

The second part of this investigation was an analysis of specific aspects of hepatic ultrastructure of the three groups of ground squirrels. In question was whether the diverse acclimation histories of the control, hibernating, and arousal groups were reflected in the hepatic rER quantitated from these ground squirrels.

Figure 4 is a transmission electron micrograph representative of those examined in the morphometrical analysis of this investigation. As indicated in Table IX, the mean V_vs (volume of rER/total volume) of the control, hibernating, and arousal groups were 9.52, 9.13, and 11.1 intersections/grid, respectively. Table X presents the results of the one factor, two tailed ANOVA performed upon these data. This evaluation indicated that there was no significant difference ($P = 0.50$) in the amount of rER quantitated from the hepatocytes of the three groups of squirrels.

TABLE IX

HEPATOCYTE VOLUME DENSITY (V_v)

Group	Controls (7)	Hibernators(6)	Arousals (6)
Mean	9.52 ± 0.358[1]	9.13 ± 1.17	11.1 ± 0.589

[1] Values are stated as mean values ± S.E.M.

TABLE X

ANOVA OF HEPATOCYTE (V_v)

	F-ratio	Probability	Result
Volume Density	1.817	0.242	NS

DISCUSSION

LIPOPROTEIN METABOLISM

As indicated in Table VI, the value of total cholesterol of the control group was 272 mg/dl. This value is lower than that reported earlier for *S. tridecemlineatus* by Galster and Morrison (1966), who sampled ground squirrels in late February and early March. Their investigation employed a combination of turbidity measurements and paper electrophoresis densitometry to determine serum lipid values of 1,100 mg/dl. These techniques, when applied individually or in combination, lack the precision and accuracy of modern electrophoretic or spectrophotometric procedures (Tietz, 1976; Isolab Bulletin, 1991). When the total plasma cholesterol concentration determined in the present investigation was compared with those of nonhibernating mammals, it was slightly higher than the value for humans, 230mg/dl (Sodeman and Sodeman, 1974), but far greater than that for the mouse, *Mus musculus*, whose average total cholesterol was 130mg/dl (Galster and Morrison, 1977).

Procedural differences notwithstanding, the study of Galster and Morrison (1966) demonstrated that the plasma lipid levels of *S. tridecemlineatus* fluctuate in a seasonal manner. During the course of one year, levels of total plasma lipids peaked in December and January, then declined gradually through June. The levels increase during the summer and autumn, peaking once again in the winter. This information implies that the

present values of the controls may have been higher had they been sampled earlier in the winter, or lower if they had been sampled in the spring.

Since small mammalian hibernators rely upon endogenous lipid stores to maintain homeostasis (Aloia and Raison, 1989), the present investigation anticipated that the total plasma concentrations of lipoproteins, as measured by cholesterol content, would increase in the hibernators and arousals relative to the controls. Such increases of the plasma lipids of hibernating ground squirrels have been documented (Galster and Morrison, 1975; Ambid, 1986; Russom *et.al.*, 1989). Taken in conjunction with a decreasing fat mass and an RQ of ~ 0.7, these predicted increases would be interpreted as evidence of increased utilization of lipid in the hibernating state.

In the hibernators, the increase of plasma β lipoproteins would have resulted from mobilization of lipid stores in an animal surviving under fasting conditions. Concurrently, α lipoprotein levels would increase since HDLs facilitate the catabolism of β lipoproteins and because HDLs may actually be formed from the by-products of β lipoprotein catabolism (Pownall *et. al.*, 1982). Similarly, it was anticipated that α and β lipoprotein levels would be elevated in the arousal group relative to the controls. The predicted increase of calorie-rich β lipoproteins would result from an increased mobilization of these lipoproteins to serve as substrates for the thermogenic brown adipose tissue (BAT) of the squirrel. BAT is a specialized form of adipose tissue which generates heat endogenously (Nizielski *et. al.*, 1989). The fuels for this process are free fatty acids and triglycerides (carried in β lipoproteins) obtained from the plasma (Cameron and Smith, 1964; Carneheim *et. al.*, 1984, 1988). As predicted for the

hibernators, α lipoproteins would increase to facilitate the catabolism β lipoproteins and from their formation from by-products of β lipoprotein catabolism.

Tables VI and VII show that the mean values of total plasma cholesterol of hibernating and arousal ground squirrels were 296 and 288mg/dl, respectively. Although these levels were elevated slightly above that of the control group, the overall difference ($P = 0.80$) was not significant ($P < 0.05$). Moreover, when the individual α and β lipoproteins comprising the total value were compared, the α lipoproteins yielded a P value of 0.40, while the P value of the β lipoproteins was 0.09 (Tables VI and VII). While not statistically significant, our data indicate a trend in which the hibernating and arousal groups exhibited increased levels of α lipoproteins and decreased levels of β lipoproteins relative to the levels of the control group.

The finding of decreased plasma β lipoproteins in the hibernating and arousal groups may seem to contradict the hypothesis of increased utilization of these lipoproteins. However, numerous investigations of mammalian lipoprotein metabolism support the hypothesis that increased catabolism of β lipoproteins may decrease their levels. For example, Carneheim *et.al.* (1984, 1988) examined the activity of lipoprotein lipase (LPL) in the brown adipose tissue (BAT) of cold exposed Sprague Dawley rats. During short-term cold exposure, the FFA reserves of BAT became depleted and the activity of BAT associated LPL became increased at least two-fold. As previously demonstrated by Cameron and Smith (1964), this resulted in decreased plasma FFAs and triglycerides (which are carried in β lipoproteins) since their rate of clearance from the plasma exceeded their rate of mobilization.

Research on the dynamics of plasma lipoprotein metabolism on human subjects under normothermic conditions is even more extensive than that on cold exposed rodents, both non-hibernating and hibernating. This research also supports the premise that increased β lipoprotein catabolism can decrease their levels. In an investigation of the interactions of VLDL and HDL in humans, Magill (1982) found that LPL activity was directly proportional to the catabolic rate of VLDL (a component of the β fraction). Increased rates of β catabolism were positively correlated with increased concentrations of HDL (α lipoproteins). It was determined that the increase of α lipoproteins resulted from decreased catabolism of α lipoproteins once they became enriched with the remnants of β lipoprotein catabolism, not from an increase in their synthesis. Future research might examine whether LPL activity is increased in hibernating and arousal groups of ground squirrels.

An additional parameter measured in this investigation was the ratio of β lipoprotein cholesterol/total cholesterol. The data from the arousal group indicates that the challenge of maintaining euthermia in the cold is responded to with a lipoprotein profile that is qualitatively different than that required in the room temperature environment of the control group. An ANOVA comparing the mean β/total cholesterol indicated that a significant difference ($P = 0.004$) existed among the three groups (Tables VI-VII). Subsequent analysis with the multiple comparison test of Tukey showed that the significance was the result of the comparison between the control group and the arousal group ($P = 0.004$). Other comparisons were not significant. Relating this information to the present investigation, the increased α and decreased β plasma

lipoproteins in the hibernating and arousal groups may be indicative of an increased utilization of β lipoprotein catabolism.

While Magill (1982) and Carneheim *et.al.* (1984, 1988) have shown that increased β lipoprotein catabolism can result in decreased β and increased α lipoprotein levels, Zech *et. al.* (1978) and Fidge *et. al.* (1980) have demonstrated, conversely, that decreased β lipoprotein catabolism can result in increased β and decreased α lipoprotein levels. Zech *et. al.* (1978) found that human subjects who produce a defective form of LPL (and consequently fail to metabolize β lipoproteins) were characterized the accumulation of plasma β lipoproteins and low levels of circulating α lipoproteins. Additionally, Fidge *et. al.* (1980) demonstrated that the catabolism of α lipoproteins was 20% greater in hypertriglyceridemic human subjects who were characterized by low LPL activity.

In the present investigation, the significant changes in hibernating ground squirrel lipid profiles noted by other authors (Galster and Morrison, 1975; Ambid, 1986; Russom *et. al.* 1989; Russom *et. al.*, 1992) were not evident. The reason for this discrepancy might be that previous studies compared winter hibernating groups with control groups consisting of spring or summer euthermic animals. When using such a method, any variations discovered may not be related necessarily to the physiology of hibernation, since the phenomenon may manifest itself in nonhibernators as well. During the "Second International Symposium on Living in the Cold", Wang (1989) noted that one of the lessons learned from the past 20 years of hibernation research was that some seasonally different functions were too readily attributed solely to the physiology of hibernation.

As an example, gonadal atrophy had been routinely considered a consequence of ground squirrel hibernation until Kenagy (1980, 1981 a,b) demonstrated that such atrophy is a seasonal phenomenon that occurs independent of the hibernating state. As such, the differences established by previous studies using post-hibernating controls may have been influenced by the type of circannual pattern of changes reported by Galster and Morrison (1966). When the current investigation removed seasonal bias by sampling all groups of squirrels during the hibernation season, the magnitude of the changes did not yield statistically significant changes, though some of the trends indicated by other authors were evident.

MORPHOMETRICS

The second portion of this investigation compared the volume density (V_v) of hepatic rER among the control, hibernating, and arousal groups of ground squirrels. Previous studies have focused on whether protein synthetic abilities or ultrastructure were modified during cold exposure and/or hibernation (Whitten and Klain, 1968; Darvish and Black, 1986; Zhegunov, 1988; and Steffen *et. al.*, 1991). The present investigation attempted to compare changes in the levels of circulating lipoproteins with the density of the hepatic rER from which they originate. The data generated indicate that there were no differences in the V_v of hepatocyte rER among the hibernating, arousal, and warm acclimated *S. tridecemlineatus*. Tables IX and X show that the mean hepatic rER V_v values of the controls, hibernators, and arousals were 9.52, 9.13, and 11.1, respectively. An ANOVA comparing the morphometric data indicated that the V_v of

hepatic rER did not differ significantly among the three groups ($P = 0.242$). The fact that there were no significant changes in hepatic rER is consistent with the lack of change of levels of plasma lipoproteins during cold adaptation.

Conclusions

The trends exhibited in the lipoprotein data suggest that the hibernating and arousal groups may have been catabolizing β lipoproteins at a greater rate than the controls. That the β/total lipoprotein ratio of the arousal group differed significantly from the control values provides support for this conclusion. However, no other comparisons of lipoprotein data yielded significant differences.

The morphometrical portion of this investigation showed that the amount of rER estimated from the hepatocytes of squirrels from the three groups did not differ significantly. That the present investigation found few changes among the three groups may be attributable to the exceptional ability of *S. tridecemlineatus* to maintain homeostasis under a broad range of environmental conditions and physiological states. Quite possibly, many of the variations found in previous investigations using spring post-hibernating controls may not have been effects of hibernation *per se*, but may have represented seasonally induced changes instead. As such, the generally invariant data collected in this investigation may have been the result of all of the sampled groups exhibiting the same seasonally dependent physiological attributes. Future investigations of lipoprotein metabolism in the cold may use this investigation as a baseline from which to operate.

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The final copies have been examined by the director of the thesis committee. The signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

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